# Simultaneous hepatic and portal vein ligation induces rapid liver hypertrophy: A study in pigs

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Abstract: Background Liver hypertrophy induced by partial portal vein occlusion (PVL) is accelerated by adding simultaneous parenchymal transection ("ALPPS procedure"). This preclinical experimental study in pigs tests the hypothesis that simultaneous ligation of portal and hepatic veins of the liver also accelerates regeneration by abrogation of porto-portal collaterals without need for operative transection. Methods A pig model of portal vein occlusion was compared with the novel model of simultaneous portal and hepatic vein occlusion, where major hepatic veins draining the portal vein–deprived lobe were identified with intraoperative ultrasonography and ligated using pledgeted transparenchymal sutures. Kinetic growth was compared, and the portal vein system was then studied after 7 days using epoxy casts of the portal circulation. Portal vein flow and portal pressure were measured, and Ki-67 staining was used to evaluate the proliferative response. Results Pigs were randomly assigned to portal vein occlusion (n = 8) or simultaneous portal and hepatic vein occlusion (n = 6). Simultaneous portal and hepatic vein occlusion was well tolerated and led to mild cytolysis, with no necrosis in the outflow vein–deprived liver sectors. The portal vein–supplied sector increased by 90 ± 22% (mean ± standard deviation) after simultaneous portal and hepatic vein occlusion compared with 29 ± 18% after PVL (P < .001). Collaterals to the deportalized liver developed after 7 days in both procedures but were markedly reduced in simultaneous portal and hepatic vein occlusion. Ki-67 staining at 7 days was comparable. Conclusion This study in pigs found that simultaneous portal and hepatic vein occlusion led to rapid hypertrophy without necrosis of the deportalized liver. The findings suggest that the use of simultaneous portal and hepatic vein occlusion accelerates liver hypertrophy for extended liver resections and should be evaluated further. Introduction Portal vein occlusion by ligation (PVL) or embolization is a well-established method to improve the safety of extended liver resections by inducing liver hypertrophy before resection.1, 2, 3 The novel procedure associating liver partition and portal vein ligation (ALPPS) indicated that kinetic growth of the future liver remnant can be increased by adding a parenchymal transection between the portal vein–supplied and portal vein–deprived part of the liver.4 A recent pig study from our laboratory suggested that the parenchymal transection in ALPPS leads to an abrogation of extensive porto-portal neocollaterals between the portal vein–supplied and the portal vein–deprived side of the liver.5 Porto-portal collaterals are common in PVL and also occur in portal vein embolization; these collaterals are known to blunt hypertrophy, and their complete abrogation may well be the cause for the astounding effectiveness of parenchymal transection in ALPPS. Based on the concept that abrogation of collaterals is a key requirement for rapid hypertrophy, we postulated that simultaneous occlusion of hepatic and portal veins may abrogate the formation of porto-portal collaterals to the deportalized side of the liver and thereby accelerate hypertrophy compared with PVL. This study tested whether simultaneous ligation of portal veins and hepatic veins (PLV+HVL) to the deportalized side of the liver would accelerate the increase in liver volume of the contralateral nondeportalized liver in a pig model. Methods Ethical statement Approval for the experiments was obtained from the Internal Animal Care and Use Committee of Rush University Medical Center (No. 15-025). The experiments were performed in compliance with the Guide for the Care and Use of Laboratory Animals by the National Research Council, 2011 edition (https://www.nap.edu/download/12910), and the ARRIVE guidelines.6

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# Simultaneous Hepatic and Portal Vein Ligation Induces Rapid Liver Hypertrophy - a Study in Pigs

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**Key words**: ALPPS, Liver regeneration, Regenerative liver surgery, Rapid hypertrophy, Portal vein ligation

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**Abbreviations**

| | |
|---|---|
| ALPPS | Associating liver partition and portal vein ligation for staged hepatectomy |
| ANOVA | Analysis of variance |
| ARRIVE | Animal Research Reporting of In Vivo Experiments |
| AST | Aspartate aminotransferase |
| ALT | Alanine aminotransferase |
| CL | Caudate liver lobe (pig) |
| CVP | Central venous pressure |
| FLR | Future liver remnant |
| IACUC | Internal Animal Care and Use Committee |
| IL6 | Interleukin-6 |
| INR | International normalized ratio |
| LL | Left lateral liver lobe (pig) |
| LM | Left median liver lobe (pig) |
| PVL | Portal vein ligation |
| PVL+ HVL | Portal vein ligation and hepatic vein ligation |
| RL | Right liver lobe (pig) |
| RM | Right medial liver lobe (pig) |
| tBili | total bilirubin |
| TNF-α | Tumor necrosis factor alpha |

**Category:** Original report


**Abstract**

**Background**: Liver hypertrophy induced by partial portal vein occlusion (PVL) is accelerated by adding simultaneous parenchymal transection ("ALPPS procedure"). This preclinical experimental study in pigs tests the hypothesis that simultaneous ligation of portal and hepatic veins (PVL+HVL) of the liver also accelerates regeneration by abrogation of porta-portal collaterals without need for surgical transection.

**Methods**: A pig model of PVL was compared to the novel simultaneous PVL+HVL, where major hepatic veins draining the portal vein deprived lobe were identified with intraoperative ultrasound and ligated using pledgeted transparenchymal sutures. Kinetic growth was compared and the portal vein system was then studied after 7 days using epoxy casts of the portal circulation. Portal vein flow and portal pressure were measured and Ki-67 staining was used to evaluate the proliferative response.

**Results:** Pigs were randomized to PVL (n=8) vs. PVL+HVL (n=6). PVL+HVL was well tolerated, led to mild cytolysis and no necrosis in the outflow vein-deprived liver sectors. The portal vein supplied sector increases by 90±22% after PVL+HVL compared to 29±18% after PVL ($p<0.001$). Collaterals to the deportalized liver developed after 7 days in both procedures, but were markedly reduced in PVL+HVL. Ki-67 staining at 7 days was comparable.

**Conclusions**: This study in pigs shows that simultaneous PVL+HVL leads to rapid hypertrophy without necrosis of the deportalized liver. The findings suggest the use of simultaneous PVL+HVL accelerates liver hypertrophy for extended liver resections should be further evaluated.

## Introduction

Portal vein occlusion by ligation (PVL) or embolization is a well established method to improve the safety of extended liver resections by inducing liver hypertrophy prior to resection.[1-3] The novel procedure Associating Liver Partition and Portal Vein Ligation (ALPPS) demonstrated that kinetic growth of the future liver remnant can be increased by adding a parenchymal transection between the portal vein supplied and portal vein deprived part of the liver.[4] A pig study from this laboratory recently suggested that the parenchymal transection in ALPPS leads to an abrogation of extensive porto-portal neo-collaterals between the portal vein supplied and the portal vein deprived side of the liver.[5] Porto-portal collaterals are common in PVL, also occur in portal vein embolization, they are known to blunt hypertrophy and their complete abrogation may well be the cause for the astounding effectiveness of parenchymal transection in ALPPS. Based on the concept that abrogation of collaterals is key for rapid hypertrophy, we postulated that simultaneous occlusion of hepatic and portal veins may abrogate the formation of porto-portal collaterals to the deportalized side of the liver and thereby accelerate hypertrophy when compared to PVL. This study tested whether simultaneous ligation of portal veins and hepatic veins (PLV+HVL) accelerates liver volume increase of the contralateral liver in a pig model.

## Methods

*Ethical statement*

Approval for the experiments was obtained from the Internal Animal Care and Use Committee (IACUC) of Rush University Medical Center (N°15-025). The experiments were performed in compliance with the 'Guide for the care and use of laboratory animals' by the National Research Council, 2011 edition (https://www.nap.edu/download/12910) and the ARRIVE guidelines.[6]

*Study design and allocation*

Pigs were randomly allocated to undergo either PVL or simultaneous PVL with HVL (PVL+HVL). The primary endpoint of the study was the increase in liver volume of the portal vein supplied part of the liver and the development of collaterals after one week. Additionally, data from a previously published study[5] were used to compare the liver volume increase after PVL and PVL+HVL with the liver volume increase in a pig model of ALPPS.

*Experimental animals and housing*

Female Yorkshire Landrace pigs were obtained from Oak Hill Genetics (Oak Hill, IL). The pigs were kept in pairs in standard pathogen-free conditions in an alternating 12h light/dark cycle at least for 7 days for accommodation. Food and water were provided ad libitum and the ambient temperature was 22±1°C. The animals were weighed prior to the procedures, and after 7 days, when they were reexplored and the livers procured. The surgical procedures were always performed in the mornings between 9:00 am and 12:00 am. After surgery the animals were housed individually to avoid tampering with their tunneled venous catheters.

*Sample size*

Based on previously published experiments we included 14 animals in the study in order to being able to study more than 5 pigs per group in the random allocation. In a previously reported study the difference in volume growth between ALPPS (n=4; 63±14%) and PVL (n=4; 16±9%) was 48% with normal distribution.[7] Assuming a power of 0.8 and a standard deviation of 0.1 and a difference to detect of 0.48, at least 5 animals per group were necessary to find out if the novel procedure PVL+ HVL induces as much hypertrophy as ALPPS. Sample size calculation was performed using JMP by SAS (Cary, N.C.,USA).

*Experimental procedures - Anesthesia*

A 25mcg fentanyl patch was placed on each pig the evening prior to surgery. On the day of surgery the animals were pre-medicated with a single intravenous (i.v.) intramuscular injection of telazol and xylazine dosed from a weight-based medication table and given atropine (0.09ml/kg, intramuscular injection). Peripheral i.v. catheters were inserted into each ear and animals were intubated using 5 mm endotrachial tubes. Animals were ventilated with a Jorvet SAV 2500 veterinary ventilator (Smiths Medical PM, Inc., Norwell, MA) at 15 breaths/minute using a Jorvet Isotec 4 nebulizer with Isoflo (Abbot Laboratories, North Chicago, IL), 1.0-3.0 vol% vaporized in 600ml oxygen/min. An open cut-down was performed to the external jugular vein to insert a tunneled 2-lumen central venous catheter (Leonhard® central venous catheter, Bard, Murray Hill, NJ) as well as an arterial line (Arrow®,Teleflex, Morrisville, NC) into the right carotid artery. Arterial pressure was monitored using a GE transport Pro monitor (GE Medical Systems, Milwaukee, WI). Volume resuscitation was maintained using a Flow Guard 6300 Dual channel volume infusion pump (Baxter, Deerfield, IL) with a strict central venous pressure at 5 mmHg to standardize hemodynamic measurements during the procedure. After the procedures, the arterial line was removed by ligation of the carotid artery and both limbs of the tunneled central venous catheter were locked with 1000U/cc heparin sulfate to prevent blood clots and allow blood draws in the postoperative period.

*Experimental procedures - Surgery*

The pig liver consists of five portal vein supply sectors (right lateral –RL, right medial –RM, left medial –LM, left lateral –LL and caudate lobe-CL) which are only partially congruous with the externally visible lobulation (**Figure 1A.**)

PVL was performed using the right lateral sector (RL) as previously described (**Figure 1B**)[5]. Ten milliliters of methylene blue was injected into the portal vein to identify the demarcation line to the RL sector prior to marking its borders with electrocautery.

HVL+PVL was performed in addition to portal vein ligation (**Figure 1C)**. The hepatic veins draining the RM, LM sector and LL sector were identified using intraoperative ultrasound (**Figure 1D**). Double armed CTX Nr. 1 prolene sutures were stitched around each vein under ultrasound guidance, quadruple-folded umbilical tape was threaded on the suture to serve as a pledget on the liver surface (**Figure 1E**) and the sutures were tied down slowly until cessation of flow could be confirmed by ultrasound (**Figure 1F**). The abdomen was then closed using Nr. 1 PDS sutures, skin was closed with 3-0 Vicryl, the lines were removed except for the double lumen Leonhard catheter, and the animals were recovered.

A laparotomy was performed 7 days later, again under general anesthesia. Ten milliliters of Methylene blue were injected into the portal vein to test for demarcation on the liver surface. After ultrasonic and photographic documentation, 2 liters of saline were used to perfuse the liver using aortic cannulation and the liver was procured.

*Primary outcome – volume increase*

The right lateral sector was weighed using Archimedes' principle of immersion weight shift using the demarcation line of the posterior sector as the immersion line as described before.[7] The future liver remnant (FLR) volume measured after 7 days was compared to an assumed baseline volume, calculated from weight of each individual pig and the median liver-to body weight index of the RL in normal pigs of 0.46 as described before.[7] Growth of the posterior liver sector after 7 days was measured directly and expressed in absolute volume increase in milliliters and also in relative increase in percent.

*Experimental outcomes - Portal Vein Casts*

Epoxy resin (Batson's Anatomical Corrosion Kit, Polysciences, Warrington, Pennsylvania) was used, red color to cast the portal vein system and blue color to cast the hepatic vein system as described before.[5] Of the eight pig livers after PVL, four casts were performed, four others were used for histological analyses of the collateral system (not reported here). Of the six pig livers after PVL+HVL, five casts of the portal vein system and one arterial cast were made. A Vernier digital microcaliper (Mitotoyu, Aurora, IL) was used to measure the diameter of collaterals.

*Experimental outcomes – hemodynamic parameters*

For hemodynamic measurements CVP was kept at 5 mmHg by graduated fluid resuscitation. Portal flow was assessed using flow probes (Transonic, Elsloo, The Netherlands) and portal vein pressure was assessed using pressure transducers as described before.[7] Portal flow was quantified as portal flow per cubic centimeter liver tissue and portal pressure was expressed as portacaval pressure gradient across the liver.

*Experimental outcomes – Laboratory values*

Blood chemistry, hematology, liver panel and coagulation parameters were determined daily as described before.[7]

*Experimental outcomes – Histology*

Liver tissue was obtained by wedge biopsy at baseline after entering the abdomen and after 7 days immediately after entering the abdomen. Tissue was fixed, embedded and stained with HE Ki-67 immunohistochemistry as described before.[7] Differently from our previous study,[7] the number of Ki-67 positive cells was determined by counting the number of Ki-67 positive

hepatocytes per 1000 hepatocytes by exclusion of Kupffer, stellate and endothelial cells.[5] Image J, NIH, Bethesda, Washington DC, was used for calibration of histology.

*Statistical methods*.

Results are expressed in mean and standard deviation parametric and median, and range for non- parametric data. Kolmogorov-Smirnov test was used for normal distribution. For comparison of groups ANOVA was used for parametric data, Kruskal-Wallis, or Friedmann tests were used for non-paired and paired non-parametric data. XY-statistics was performed using linear regression. P values $< 0.05$ were considered significant. Details on descriptive and comparative statistics used are given in each figure legend. Prism 6.0 (GraphPad Software Inc. San Diego, CA) was used for data analysis and presentation.

## Results

*Outcome: Volume changes*

Absolute volume increase 7 days after the respective procedures for each animal is shown in **Figure 2A** with the mean increase marked in bold. **Figure 2B** shows the same volume increase in percent. The portal vein supplied sector increases by 29±18% after PVL, but by 90±22% after PVL+HVL. Compared to baseline volume, the increase 7 days after PVL+HVL is significant ($p<0.001$), while the volume increase 7 days after PVL is not. There is a difference between PVL+HVL and PVL at 7 days by the factor of 3.1 ($p<0.001$). The liver deprived of portal flow remains unchanged after PVL, but collapses significantly in volume after PVL+HVL (**Figure 2C**). **Figure 2D** shows the volume change of the deportalized sectors for each pig in percent with the mean in bold. The volume loss after PVL+HVL was consistent and at a mean of 18%(±8), while after PVL some of the livers deprived of portal flow increased, others decreased in volume relative to day 0.

*Outcome: Liver surface staining with methylene-blue*

At the time of surgery at day 0 the injection of methylene blue showed the characteristic sector demarcation going across the two external lobulations (**Figure 3A**). At the time of surgery at day 7, a blue demarcation line was largely absent in PVL, there was staining across the liver with no clear demarcation line (**Figure 3B**). In contrast, RL+CL stained intensely 7 days after PVL+HVL with the demarcation line shifted somewhat medially **(Figure 3C).**

*Outcome: Epoxy casting of the portal vein system*

A representative example of 4 epoxy casts 7 days after PVL is shown in **Figure 4A.** The portal vein system of the portal vein deprived liver, although completely disconnected from the main portal vein, filled from the portal vein system supplying the RL+CL sector through dense collaterals, each with a diameter between 0.5 to 4 mm, across the watershed to the left portal vein system as reported before.[7] Three systems of collaterals were identified, one anterior to the right hepatic vein **(Figure 4B, one arrow)**, one posterior to the right hepatic vein **(Figure 4 B, two arrows)** and one through the caudate lobe **(Figure 4B, three arrows)**. In the epoxy cast model 7 days after PVL/HVL, collaterals had also developed across the watershed, but smaller and less pronounced **(Figure 4C)**. All 3 collateral systems that were observed in PVL were present, but with less and smaller sized collaterals. Collaterals anterior **(Figure 4D, one arrrow)** and posterior **(Figure 4D, two arrows)** to the right hepatic vein were found, as well as through the caudate lobe that wraps the vena cava and connects the RL to the RM lobe in pigs **(Figure 4 E, three arrows)**. Quantification of collateral development after seven days was performed by counting the number of collaterals larger than 1 mm in diameter and plotting them on a correlation graph (**Figure 4E**). There is a good correlation

(R=0.83, p<0.001) between the number of collaterals > 1mm and the volume increase in volume of the portal vein supplied sector within seven days.

*Outcome: Hemodynamic changes in PVL and PVL+HVL*

Portal vein volume flow measurements prior to and 1 hour after performing the ligations of PVL or PVL+HVL, showed no significant difference in main portal vein flow after one hour or seven days later, when the livers were procured. This indicates that in pigs, the same mesenteric blood volume is pushed through the portal supplied part of the liver after the ligation procedures as is flowing through the entire liver before, as has been demonstrated in previous experiments in our laboratory in rats[8] and pigs.[7] The index of volume of portal blood flow (in ml) per volume liver tissue (in cc) per minute increased from 1.06 (IQR0.83-1.25) and 1.29 (IQR 1.14-1.36) ml/cc/min to 3.69 (IQR2.93-5.39) and 5.68 (IQR4.61-5.96) ml/cc/min in PVL and PVL+HVL, respectively after the ligation procedures on day 0. There is no difference in the flow changes between PVL and PVL+HVL. The index remains elevated after 7 days, if the growing portal vein supplied liver remnant is in the denominator as shown in **Figure 5A**. If the portal blood however perfuses a larger part of the liver as suggested by the collaterals found in the epoxy casts, the denominator increases in a way that is difficult to determine and the true index is difficult to assess. The gradient between central vein pressure (CVP) and portal vein pressure in pigs ("portacaval gradient") in mmHg at baseline is 1 (IQR1-2.75) mmHg and 2.5 (IQR1.75-3) mmHg and increases to 6.5 (IQR5.25-7.75) mmHg and 5.5 mmHg (IQR4.75-9.25) for PVL and PVL+HVL, respectively (p=0.008 and p= 0.007, *resp*.) after the flow restriction to about 21% of the previous liver volume (**Figure 5B**). The acute increase of the pressure gradient returns to normal after 7 days in both procedure types. There is no difference in the acute portal hypertension induced by PVL and PVL+HVL.

*Outcome: Serum hepatocyte injury markers and liver function tests*

The animals were well, no animal died prematurely and no animal had ascites on reexploration . The deportalized and the growing liver looked viable on reexploration on day 7 in both groups. **Figure 5C** shows that aspartate aminotransferase (AST) levels are increased (p=0.004) after both PVL and double ligation PVL+HVL, remain so until day 3, when they return to normal. There is no significant difference between PVL and PVL+HVL. Alanine aminotransferase (ALT) remains unchanged and does not differ between procedures **(Figure 5D)**. The liver transfer function metrics total bilirubin (tBili) is very low in pigs at baseline and remains unchanged **(Figure 5E)**. Liver synthetic function metrics International Normalized Ratio (INR) and PT also remained unchanged and does not differ between procedures **(Figure 5F)**.

*Outcome: Histologic changes*

**Figure 6A** shows the normal portal tract anatomy in pigs with normal sized portal veins. **Figure 6B** and **Figure 6C** show dilated portal veins 7 days in the portal vein supplied growing lobes after PLV and PVL+HVL respectively. Quantitative evaluation of Ki-67 staining at baseline **(Figure 6D)**, 7 days after PVL **(Figure 6E),** and 7 days after PVL+HVL **(Figure 6F)** show no significant changes **(Figure 6G)**. The liver lobe deprived of portal inflow and venous outflow does not show any signs of tissue degeneration in 100x magnification **(Figure 6H)** or inflammatory infiltrates or cellular degeneration in 600x magnification **(Figure 6I)**.

**Discussion**

This study demonstrates in a preclinical animal model that the simultaneous double ligation of portal and hepatic veins induces three times the amount of liver hypertrophy compared to portal vein ligation alone within 7 days. This simultaneous double ligation maneuver is surprisingly well tolerated, does not lead to excessive transaminase elevation when compared to portal vein ligation alone and does not produce any laboratory evidence of liver dysfunction as evidenced by serum tBili or INR. Despite the outflow obstruction of all major veins, there is no evidence of hepatocyte necrosis in histology, likely due to remaining arterial flow. The amount of hypertrophy induced by this double ligation technique is at least as extensive as the rapid hypertrophy in a pig model of ALPPS with 64 % volume increase within 7 days.[7] Both methods lead to comparable volume increase in the portal vein supplied sector compared to PVL alone.

The mechanism of the observed effect of double ligation on hypertrophy remaine unclear. We speculate that the inflow of hepatotrophic growth factors through collaterals can be abrogated either by transection of the parenchyma or by additional ligation of the hepatic veins additional to the portal veins. We propose that the mechanism is abrogation of collateral flow bringing the substances that have in the past been described as the "hepatotrophic growth factors" like insulin, insultin-like growth factor, epithelial growth factors etc. to the growing liver. In the ALPPS model in pigs the area of portal vein perfusion in ALPPS after 7 days as indicated by methylene blue coloring had been confined to the right lobe along the line of parenchymal transsection (not shown).[7] Similarly now, 7 days after PVL+HVL, the area of portal vein perfusion stayed restricted to too the right lobe **(Figure 3C)** while it dissipated across the entire liver in PVL **(Figure 3B).**

The hypertrophy of the double ligation approach in a preclinical pig model is surprising, since sequential hepatic vein occlusion has in the past been found to be only moderately effective, likely because hepatic vein occlusion has been used previously as a salvage maneuver in a sequential fashion after portal vein occlusion.[9, 10] The profound effect on volume may be the result of the simultaneous occlusion of both systems.[11, 12] It may be that an initial growth stimulus matters without portal escape collaterals matter and sequential embolisation does not provide that. Interestingly also, simultaneous portal vein and hepatic vein occlusion has been attempted in preclinical rabbit model in the past, but was found to yield a hypertrophy response that was not considered superior to portal vein embolization alone.[13] The findings may be explained by the anatomic specificities of the rabbit, i.e. the almost complete disconnection of the parenchyma in the highly lobulated rabbit liver.[14] The anatomy in the rabbit liver does not lend itseld to study the effects of collateral abrogation because the liver is much more extensively lobulated than the pig liver. After embolization or ligation of the left or distal liver the potential for development of collaterals is minimalm because there is only a diminutive parenchymal tissue mass connecting the deportalized and the growing liver. The disappointing findings in patients and animal model may have discouraged others from pursuing a simultaneous double occlusion of both portal and hepatic veins.

Recently however, the simultaneous embolization (rather than ligation) of portal and hepatic veins has been described in humans in a pilot series under the name of "liver venous deprivation" technique (LVD) with results that suggest that simultaneous double embolization in humans also induces rapid hypertrophy.[11, 12]

While ALPPS currently yields a lot of attention because it allows induction of rapid hypertrophy prior to resection with the promise to expand resectability of liver tumors with large tumor load,[4, 15, 16] ALPPS as a routine procedure remains controversial.[17-19] ALPPS has a high perioperative morbidity and mortality rate.[20, 21] Recently improvements in outcomes

have been described, both in highly selected patient populations[22] and in prospective series with more restrictive inclusion criteria[23]. There is accumulating evidence however that ALPPS produces a large volume of immature liver tissue in a short period of time that is voluminous but dysfunctional and may put patients at risk of post-hepatectomy liver failure more often than predicted by the large volume increase.[24-28] In contrast, there is now preliminary evidence in three patients that simultaneous double embolization in humans does not induce a function/volume incongruence.[11] It is an interesting finding that rapid hypertrophy comparable in magnitude to ALPPS does not necessarily result in a functional deficit. The understanding of the physiological elements associated with rapid hypertrophy remain an important task to clarify whether the circulating growth factors like Il-6 and TNF-α, the acute portal hypertension or the difference in the extent of abrogation of collaterals ultimately explain the volume increase and also the occurrence of the functional deficit after classic ALPPS and how functionality can me maintained in rapid hypertrophy. This large animal model confirms that rapid volume increase occurs after double ligation and will allow to study questions of volume and function by comparing PVL, the ALPPS model and double ligation..

There is evidence that the number of collaterals negatively correlates with the degree of hypertrophy in PVL and ALPPS.[7] Additionally, there are case reports on failed hypertrophy due to developing porto-portal collaterals after PVE in humans.[29] There also is the presumption that PVE is more effective than PVL in humans because of reduced collateralization in general when the portal venous space is filled with glue.[30] Going back almost 100 years, Peyton Rous observed collaterals to the portal vein deprived liver in the first paper on experimental portal vein ligation in 1921.[31] He noticed that *"The rapidity and*

*completeness of the atrophy is in our experiments proportionate to the number of these little collaterals. Their influence may be directly seen where they enter the liver."*[31]

In the epoxy casts a reduction, not a complete absence of collaterals in the double ligation model was found. Both models induced acute portal hypertension and in both models gradients normalized one week after ligation. It has to be emphasized that hepatic veins, while occluded by US immediately after the performance of PVL+ HVL, had partially recanalized at day 7. These observations warrant future studies on the hemodyamics of collateral flow along the time axis.

The histologic findings of this study and the comparable Ki-67 proliferation rates of hepatocytes are different from our findings in the ALPPS model in pigs[7], and suggest that the process of hepatocyte proliferation in PLV+HVL in pigs is terminated prior to day 7. Evaluations at earlier time points should address this point in the future.

A methodological limitation of this study is the lack of cross-sectional imaging in our large animal facility to allow a detailed diachronous assessment of volume changes and possibly also an interrogation of in vivo inflow kinetics using contrast. Also, an assessment of liver function by regional liver function test using hepatobiliary iminodiacetic acid (HIDA) scanning was not yet possible in this study due to the absence of a gamma-camera or SPECT computer tomography for large animals. Combined volume and function assessment would greatly enhance the understanding of the different types of liver hypertrophy und their functionality. Differently from the human studies on double embolization this large animal model allows an interrogation of the vascular anatomy by performing epoxy vascular casts. These casts provide a static snapshot of anatomic detail of the collaterals developed, that has not been demonstrated by any other study so far.

Another limitation is that PVL+HVL is compared to PVL alone, but not directly also to ALPPS or PVE. In a large animal model the three Rs (reduce, refine, replace) of laboratory

animal science led us to not repeat the previously published ALPPS series again but compare the new results with PVL+HVL to data published about ALPPS in the pig.[7] The allocation to two groups, PVL vs. HVL+PLV is a simple study design. The power analysis for sample size was performed based on data from our previously published study of PVL vs. ALPPS. The randomization resulted in an asymmetric allocation of 8 vs. 6 animals, which is methodologically unavoidable. In the future, PVE in the pig and also hepatic vein embolization in this laboratory will be tested but they require a new experimental set up with fluoroscopy and involvement of interventional radiologists.

In summary, this preclinical study demonstrates the feasibility and safety of simultaneous unilateral occlusion of hepatic and portal veins to induce rapid liver regeneration. It maybe the case that the double venous ligation or embolization technique in man allows for a safer acceleration of hypertrophy than ALPPS has so far. Double ligation and embolization should be further evaluated with careful analyses of liver function to evaluate this potential alternative to ALPPS in regenerative liver surgery.

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## Figure Legends

### Figure 1

**Experimental procedures in pigs. (A) Portal vein anatomy of the pig liver**. The right lateral sector (**RL**), caudate lobe sector(**CL**), right median sector (**RM**), left median sector (**LM**) and left lateral sector (**LL**) are shown. Portal vein supply sectors are not congruent with the borders of the external lobulation, a common theme in liver anatomy. The watersheds between sectors cross the lobes and tissue bridges between lobes in a sigmoid line (dotted line). **(B) Portal vein ligation (PVL) procedure** PVL leads to an acute cessation of portal vein flow to RM, LM and LL and portal hyperflow to RL and CL. The ligation of the portal vein distal to the take-off of the RL lobe branch (arrow) is achieved using portal pedicle dissection and a silk tie. **(C) Portal vein ligation + hepatic vein ligation procedure (PVL+HVL**). In PVL+HVL, additionally to PVL the three hepatic veins draining RM, LM and LL are additionally ligated using long and large XLS needles with Nr. 1 Prolene sutures and pledgets. The RM, LM and LL veins are ligated. **(D) Intraoperative ultrasound** is used to identify the three veins draining RM, LM and LL. A sponge is placed between the right lobe and the right median lobe to delineate a line of echogenicity between the lobes (white arrows) to safely spare the RL vein. (**E) Intraoperative photograph of PVL+HVL**. The photo shows the representative placement of a venous occlusion stitch with the white umbilical tape serving as a pledget. The three inserts show the steps of the procedure, the depth of the needle tract is determined by ultrasound. (**F**) **Photograph of intraoperative ultrasound**. At the end of the PVL and HVL procedure intraoperative ultrasound confirms the occlusion of the RM, LM and LL veins and a patent RL vein that drains into the vena cava.

**Figure 2**

**Volume changes of the pig liver over 7 days after portal vein ligation (PVL) and double ligation of portal vein and hepatic veins (PVL+HVL). (A) Absolute volume changes of the portal vein supplied sector (right lateral and caudate lobe – RL+CL) in cubic centimeters over 7 days.** The volume of the portal vein supplied sector (RL+CL) increases significantly after PVL+HVL (°p< 0.001), but not significantly after PVL (p=0.14). The difference in size between the portal vein supplied lobe 7 days after PVL and 7 days after PVL+HVL is significant (*p<0.001). Data are normally distributed by Kolmogorov-Smirnov test and one-way ANOVA was used for comparison of 4 groups. **(B) Relative volume change in the portal vein supplied sector in percent.** The graph shows the relative volume increase of the portal vein supplied sector after both procedures compared to baseline. **C) Absolute volume changes in portal vein deprived sectors (right median, left median and left lateral sector – RM+LM+LL).** The portal vein deprived liver (RM+LM+LL) reduces significantly in size after PVL+HVL (p<0.001), while it does not change significantly after PLV alone (p=0.10). There is a significant difference between the size of the deportalized sector after PVL and PVL+HVL (p<0.001). Data are normally distributed by Kolmogorov-Smirnov test and one-way ANOVA was used for comparison of 4 groups. **(D) Relative volume change in the portal vein deprived sectors in percent.** The graph shows the collapse of the volume of the portal vein deprived liver sectors after PVL+ HVL when compared to PVL.

**Figure 3 Methylene-blue staining after injection of 10 ml methylene blue into the main portal vein (A) at baseline, (B) 7 days after PVL and (C) 7 days after double ligation of portal vein and hepatic veins**. **(A)** The sigmoid shape of the portal supply area of right lateral and caudate lobe sector (RL+CL) is delineated by the blue staining of the watershed.

**(B)** There is no intraoperative line of demarcation 7 days after PVL since the methylene blue distributes across the entire liver, likely through the collaterals that have developed. **(C)** There is a line of demarcation after PVL +HVL, somewhat shifted to the right median sector when compared to the baseline in (A).

**Figure 4**

**Evaluation of portal vein collaterals** using expoxy casts of the portal vein system (red resin) and the hepatic veins (blue resin) of entire pig livers 7 days after portal vein ligation (PVL) and double ligation of portal and hepatic veins (PVL+HVL) **(A) Epoxy cast of pig liver 7 days after PVL** shows the right lateral sector (RL), the right median sector (RM), the left median (LM) and the left lateral sector (LL) of the pig liver with the blue resin as the haptic veins and the red resin the portal vein system. There are extensive neocollaterals (red resin) across the normally avascular watershed between the RL and the RM giving rise to secondary filling of the portal vein tree of RM, LM and LL. The vena cava (blue) is deflected posteriorly and marked by asterisk (*). The square frames the enlarged area in Figure 4 (B). **(B) The enlargement of the frame in 4A** shows the 3 main groups of collaterals observed in the PVL cast, (one arrow, red) anterior to the RL vein, (two arrows, red ) posterior to the RL vein and (three arrows, red) through the caudate lobe. **(C) Epoxy cast of pig liver 7 days after PVL+HVL.** The extensive neocollateralization between RL and RM observed after PVL is here notably absent except for very few collaterals. The vena cava (blue) is deflected inferiorly and marked by asteriks (*). The 3 pledgets made from umbilical tape and used to reinforce the venous ligation stitches on the liver surface are visible even after the digestion process of the liver tissue. The ligated hepatic however have recanalized. The square frames the enlarged area in figure 4D. **(D) The enlargement of the frame in 4C** shows one large > 1mm diameter neocollateral (red) giving off multiple small branches (one arrow). There is

also a group of collaterals posterior to the RL hepatic vein (red, two arrows). **(E) The caudate lobe collaterals 7 days after PVL+HVL** are best visualized from an inferior perspective (red, three arrows) **(F) Correlation graph of collaterals measured by a Vernier digital microcaliper to be >1mm and percent volume increase** in 4 epoxy casts from pig livers 7 days after PVL (yellow) and 6 epoxy casts from pig livers 7 days after PVL+HVL (blue). There is a significant negative correlation (number of glomerular tufts = - 0.1605 * % hypertrophy + 18.99) with a $R^2$ of 0.83.

**Figure 5**

**Hemodynamic and laboratory changes after Portal Vein Ligation (PVL) and Double Ligation of Portal Veins and Hepatic Veins. (A) Volume flow in the main portal vein** corrected for the volume of liver tissue perfused. Intraoperative direct measurements of main portal vein flow corrected for the estimated tissue volume perfused based on estimated liver weight shows an increase in portal vein flow per tissue immediately after the procedure for both PVL (p=0.017) and PVL+HVL (p= 0.002). There are no differences between PVL and PVL+HVL for all measurements. The perfused volume after 7 days was the weighed hypertrophied liver. Given the findings of collaterals which may well recruit portal volume flow to the side that was deprived of portal flow during the procedure, a correct estimation the perfused volume at day 7 is difficult. **(B) Pressure gradient between in the main portal vein and the inferior vena cava** was measured intraoperatively by using 18 gauge intra-portal needles. The results demonstrate an acute portal hypertension as a result of both devascularization procedures PVL and PVL+HVL. In both procedures the portocaval gradients return to normal after 7 days. Friedman test for repeated measurements of non-parametric data is used, Kruskal-Wallis test is used for comparison of groups. **(C) Asparatate transferase (AST)** levels before and after the procedure at day 0, at day1 and every other day

until day 7. There is no significant increase on day 0 after PVL, but after PVL+HVL ($p<0.001$), but no difference between groups. **(D) Alanine transferase (ALT)** levels. **(E) Total bilirubin (tbili)** levels. **(F) International Normalized Ratio (INR)** levels. There is no significant difference between time points or group for ALT, AST, tbili or INR. For all statistics in this figure, Friedman test for repeated measures of non-parametric data was used. Kruskal-Wallis test was used for comparison of groups.

**Figure 6**

**Histology 7 days after Portal vein ligation (PVL) and double ligation of portal veins and hepatic veins (PVL+HVL). (A) Normal portal triad of a pig liver** at day 0 prior to surgery shows normal sized portal vein (arrow), artery and bile duct (x200 magnification). **(B) Portal triad 7 days after PVL** shows dilated portal veins (arrows) with normal bile ducts and artery (x200 magnification). **(C) Portal triad 7 days after PVL+HVL** shows a dilated portal vein (arrow) (x200 magnification). **(D) Ki-67 immunohistochemistry of a normal pig liver** at day 0 prior to surgery with staining of hepatocytes (arrow), occasional Kupffer cells (short arrow) (x400 magnification). **(E) Ki-67 immunohistochemistry 7 days after PVL** with staining of hepatocytes (arrow), occasional Kupffer (short arrow) and endothelial cells (thin arrow) (x400 magnification). **(F) Ki-67 immunohistochemistry 7 days after PVL+HVL** with a very similar appearance, staining of hepatocytes (arrow), occasional Kupffer (short arrow) and endothelial cells (thin arrow) (x400 magnification). **(G) Quantitative evaluation of Ki-67 staining between PVL and PVL+HVL.** No difference was detected. Friedman test for repeated measures of non-parametric data was used. Kruskal-Wallis test was used for comparison of groups. **(H) No evidence of necrosis** or degeneration of histologic architecture in the portal vein and hepatic vein deprived lobe 7 days after PVL+HVL (magnification x100) **(I) No evidence of necrosis**, cytoplasmic degeneration or inflammation 7 days after

PVL+HVL in higher magnification x600. *Each bar in the lower right corner represents 100 microns which was calibrated according to magnification using ImageJ*.

# Simultaneous Hepatic and Portal Vein Ligation Induces Rapid Liver Hypertrophy - a Study in Pigs

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**Abbreviations**

| | |
|---|---|
| ALPPS | Associating liver partition and portal vein ligation for staged hepatectomy |
| ANOVA | Analysis of variance |
| ARRIVE | Animal Research Reporting of In Vivo Experiments |
| AST | Aspartate aminotransferase |
| ALT | Alanine aminotransferase |
| CL | Caudate liver lobe (pig) |
| CVP | Central venous pressure |
| FLR | Future liver remnant |
| IACUC | Internal Animal Care and Use Committee |
| IL6 | Interleukin-6 |
| INR | International normalized ratio |
| LL | Left lateral liver lobe (pig) |
| LM | Left median liver lobe (pig) |
| PVL | Portal vein ligation |
| PVL+ HVL | Portal vein ligation and hepatic vein ligation |
| RL | Right liver lobe (pig) |
| RM | Right medial liver lobe (pig) |
| tBili | total bilirubin |
| TNF-α | Tumor necrosis factor alpha |

**Category:** Original report


**Abstract**

**Background**: Liver hypertrophy induced by partial portal vein occlusion (PVL) is accelerated by adding simultaneous parenchymal transection ("ALPPS procedure"). This preclinical experimental study in pigs tests the hypothesis that simultaneous ligation of portal and hepatic veins (PVL+HVL) of the liver also accelerates regeneration by abrogation of porta-portal collaterals without need for surgical transection.

**Methods**: A pig model of PVL was compared to the novel simultaneous PVL+HVL, where major hepatic veins draining the portal vein deprived lobe were identified with intraoperative ultrasound and ligated using pledgeted transparenchymal sutures. Kinetic growth was compared and the portal vein system was then studied after 7 days using epoxy casts of the portal circulation. Portal vein flow and portal pressure were measured and Ki-67 staining was used to evaluate the proliferative response.

**Results:** Pigs were randomized to PVL (n=8) vs. PVL+HVL (n=6). PVL+HVL was well tolerated, led to mild cytolysis and no necrosis in the outflow vein-deprived liver sectors. The portal vein supplied sector increases by 90±22% after PVL+HVL compared to 29±18% after PVL ($p<0.001$). Collaterals to the deportalized liver developed after 7 days in both procedures, but were markedly reduced in PVL+HVL. Ki-67 staining at 7 days was comparable.

**Conclusions**: This study in pigs shows that simultaneous PVL+HVL leads to rapid hypertrophy without necrosis of the deportalized liver. The findings suggest the use of simultaneous PVL+HVL accelerates liver hypertrophy for extended liver resections should be further evaluated.

## Introduction

Portal vein occlusion by ligation (PVL) or embolization is a well established method to improve the safety of extended liver resections by inducing liver hypertrophy prior to resection.[1-3] The novel procedure Associating Liver Partition and Portal Vein Ligation (ALPPS) demonstrated that kinetic growth of the future liver remnant can be increased by adding a parenchymal transection between the portal vein supplied and portal vein deprived part of the liver.[4] A pig study from this laboratory recently suggested that the parenchymal transection in ALPPS leads to an abrogation of extensive porto-portal neo-collaterals between the portal vein supplied and the portal vein deprived side of the liver.[5] Porto-portal collaterals are common in PVL, also occur in portal vein embolization, they are known to blunt hypertrophy and their complete abrogation may well be the cause for the astounding effectiveness of parenchymal transection in ALPPS. Based on the concept that abrogation of collaterals is key for rapid hypertrophy, we postulated that simultaneous occlusion of hepatic and portal veins may abrogate the formation of porto-portal collaterals to the deportalized side of the liver and thereby accelerate hypertrophy when compared to PVL. This study tested whether simultaneous ligation of portal veins and hepatic veins (PLV+HVL) accelerates liver volume increase of the contralateral liver in a pig model.

## Methods

*Ethical statement*

Approval for the experiments was obtained from the Internal Animal Care and Use Committee (IACUC) of Rush University Medical Center (N°15-025). The experiments were performed in compliance with the 'Guide for the care and use of laboratory animals' by the National Research Council, 2011 edition (https://www.nap.edu/download/12910) and the ARRIVE guidelines.[6]

*Study design and allocation*

Pigs were randomly allocated to undergo either PVL or simultaneous PVL with HVL (PVL+HVL). The primary endpoint of the study was the increase in liver volume of the portal vein supplied part of the liver and the development of collaterals after one week. Additionally, data from a previously published study[5] were used to compare the liver volume increase after PVL and PVL+HVL with the liver volume increase in a pig model of ALPPS.

*Experimental animals and housing*

Female Yorkshire Landrace pigs were obtained from Oak Hill Genetics (Oak Hill, IL). The pigs were kept in pairs in standard pathogen-free conditions in an alternating 12h light/dark cycle at least for 7 days for accommodation. Food and water were provided ad libitum and the ambient temperature was 22±1°C. The animals were weighed prior to the procedures, and after 7 days, when they were reexplored and the livers procured. The surgical procedures were always performed in the mornings between 9:00 am and 12:00 am. After surgery the animals were housed individually to avoid tampering with their tunneled venous catheters.

*Sample size*

Based on previously published experiments we included 14 animals in the study in order to being able to study more than 5 pigs per group in the random allocation. In a previously reported study the difference in volume growth between ALPPS (n=4; 63±14%) and PVL (n=4; 16±9%) was 48% with normal distribution.[7] Assuming a power of 0.8 and a standard deviation of 0.1 and a difference to detect of 0.48, at least 5 animals per group were necessary to find out if the novel procedure PVL+ HVL induces as much hypertrophy as ALPPS. Sample size calculation was performed using JMP by SAS (Cary, N.C.,USA).

*Experimental procedures - Anesthesia*

A 25mcg fentanyl patch was placed on each pig the evening prior to surgery. On the day of surgery the animals were pre-medicated with a single intravenous (i.v.) intramuscular injection of telazol and xylazine dosed from a weight-based medication table and given atropine (0.09ml/kg, intramuscular injection). Peripheral i.v. catheters were inserted into each ear and animals were intubated using 5 mm endotrachial tubes. Animals were ventilated with a Jorvet SAV 2500 veterinary ventilator (Smiths Medical PM, Inc., Norwell, MA) at 15 breaths/minute using a Jorvet Isotec 4 nebulizer with Isoflo (Abbot Laboratories, North Chicago, IL), 1.0-3.0 vol% vaporized in 600ml oxygen/min. An open cut-down was performed to the external jugular vein to insert a tunneled 2-lumen central venous catheter (Leonhard® central venous catheter, Bard, Murray Hill, NJ) as well as an arterial line (Arrow®,Teleflex, Morrisville, NC) into the right carotid artery. Arterial pressure was monitored using a GE transport Pro monitor (GE Medical Systems, Milwaukee, WI). Volume resuscitation was maintained using a Flow Guard 6300 Dual channel volume infusion pump (Baxter, Deerfield, IL) with a strict central venous pressure at 5 mmHg to standardize hemodynamic measurements during the procedure. After the procedures, the arterial line was removed by ligation of the carotid artery and both limbs of the tunneled central venous catheter were locked with 1000U/cc heparin sulfate to prevent blood clots and allow blood draws in the postoperative period.

*Experimental procedures - Surgery*

The pig liver consists of five portal vein supply sectors (right lateral –RL, right medial –RM, left medial –LM, left lateral –LL and caudate lobe-CL) which are only partially congruous with the externally visible lobulation (**Figure 1A.**)

PVL was performed using the right lateral sector (RL) as previously described (**Figure 1B**)[5]. Ten milliliters of methylene blue was injected into the portal vein to identify the demarcation line to the RL sector prior to marking its borders with electrocautery.

HVL+PVL was performed in addition to portal vein ligation (**Figure 1C)**. The hepatic veins draining the RM, LM sector and LL sector were identified using intraoperative ultrasound (**Figure 1D**). Double armed CTX Nr. 1 prolene sutures were stitched around each vein under ultrasound guidance, quadruple-folded umbilical tape was threaded on the suture to serve as a pledget on the liver surface (**Figure 1E**) and the sutures were tied down slowly until cessation of flow could be confirmed by ultrasound (**Figure 1F**). The abdomen was then closed using Nr. 1 PDS sutures, skin was closed with 3-0 Vicryl, the lines were removed except for the double lumen Leonhard catheter, and the animals were recovered.

A laparotomy was performed 7 days later, again under general anesthesia. Ten milliliters of Methylene blue were injected into the portal vein to test for demarcation on the liver surface. After ultrasonic and photographic documentation, 2 liters of saline were used to perfuse the liver using aortic cannulation and the liver was procured.

*Primary outcome – volume increase*

The right lateral sector was weighed using Archimedes' principle of immersion weight shift using the demarcation line of the posterior sector as the immersion line as described before.[7] The future liver remnant (FLR) volume measured after 7 days was compared to an assumed baseline volume, calculated from weight of each individual pig and the median liver-to body weight index of the RL in normal pigs of 0.46 as described before.[7] Growth of the posterior liver sector after 7 days was measured directly and expressed in absolute volume increase in milliliters and also in relative increase in percent.

*Experimental outcomes - Portal Vein Casts*

Epoxy resin (Batson's Anatomical Corrosion Kit, Polysciences, Warrington, Pennsylvania) was used, red color to cast the portal vein system and blue color to cast the hepatic vein system as described before.[5] Of the eight pig livers after PVL, four casts were performed, four others were used for histological analyses of the collateral system (not reported here). Of the six pig livers after PVL+HVL, five casts of the portal vein system and one arterial cast were made. A Vernier digital microcaliper (Mitotoyu, Aurora, IL) was used to measure the diameter of collaterals.

*Experimental outcomes – hemodynamic parameters*

For hemodynamic measurements CVP was kept at 5 mmHg by graduated fluid resuscitation. Portal flow was assessed using flow probes (Transonic, Elsloo, The Netherlands) and portal vein pressure was assessed using pressure transducers as described before.[7] Portal flow was quantified as portal flow per cubic centimeter liver tissue and portal pressure was expressed as portacaval pressure gradient across the liver.

*Experimental outcomes – Laboratory values*

Blood chemistry, hematology, liver panel and coagulation parameters were determined daily as described before.[7]

*Experimental outcomes – Histology*

Liver tissue was obtained by wedge biopsy at baseline after entering the abdomen and after 7 days immediately after entering the abdomen. Tissue was fixed, embedded and stained with HE Ki-67 immunohistochemistry as described before.[7] Differently from our previous study,[7] the number of Ki-67 positive cells was determined by counting the number of Ki-67 positive

hepatocytes per 1000 hepatocytes by exclusion of Kupffer, stellate and endothelial cells.[5] Image J, NIH, Bethesda, Washington DC, was used for calibration of histology.

*Statistical methods*.

Results are expressed in mean and standard deviation parametric and median, and range for non- parametric data. Kolmogorov-Smirnov test was used for normal distribution. For comparison of groups ANOVA was used for parametric data, Kruskal-Wallis, or Friedmann tests were used for non-paired and paired non-parametric data. XY-statistics was performed using linear regression. P values $< 0.05$ were considered significant. Details on descriptive and comparative statistics used are given in each figure legend. Prism 6.0 (GraphPad Software Inc. San Diego, CA) was used for data analysis and presentation.

## Results

*Outcome: Volume changes*

Absolute volume increase 7 days after the respective procedures for each animal is shown in **Figure 2A** with the mean increase marked in bold. **Figure 2B** shows the same volume increase in percent. The portal vein supplied sector increases by 29±18% after PVL, but by 90±22% after PVL+HVL. Compared to baseline volume, the increase 7 days after PVL+HVL is significant ($p<0.001$), while the volume increase 7 days after PVL is not. There is a difference between PVL+HVL and PVL at 7 days by the factor of 3.1 ($p<0.001$). The liver deprived of portal flow remains unchanged after PVL, but collapses significantly in volume after PVL+HVL (**Figure 2C**). **Figure 2D** shows the volume change of the deportalized sectors for each pig in percent with the mean in bold. The volume loss after PVL+HVL was consistent and at a mean of 18%(±8), while after PVL some of the livers deprived of portal flow increased, others decreased in volume relative to day 0.

*Outcome: Liver surface staining with methylene-blue*

At the time of surgery at day 0 the injection of methylene blue showed the characteristic sector demarcation going across the two external lobulations (**Figure 3A**). At the time of surgery at day 7, a blue demarcation line was largely absent in PVL, there was staining across the liver with no clear demarcation line (**Figure 3B**). In contrast, RL+CL stained intensely 7 days after PVL+HVL with the demarcation line shifted somewhat medially **(Figure 3C).**

*Outcome: Epoxy casting of the portal vein system*

A representative example of 4 epoxy casts 7 days after PVL is shown in **Figure 4A.** The portal vein system of the portal vein deprived liver, although completely disconnected from the main portal vein, filled from the portal vein system supplying the RL+CL sector through dense collaterals, each with a diameter between 0.5 to 4 mm, across the watershed to the left portal vein system as reported before.[7] Three systems of collaterals were identified, one anterior to the right hepatic vein **(Figure 4B, one arrow)**, one posterior to the right hepatic vein **(Figure 4 B, two arrows)** and one through the caudate lobe **(Figure 4B, three arrows)**. In the epoxy cast model 7 days after PVL/HVL, collaterals had also developed across the watershed, but smaller and less pronounced **(Figure 4C)**. All 3 collateral systems that were observed in PVL were present, but with less and smaller sized collaterals. Collaterals anterior **(Figure 4D, one arrrow)** and posterior **(Figure 4D, two arrows)** to the right hepatic vein were found, as well as through the caudate lobe that wraps the vena cava and connects the RL to the RM lobe in pigs **(Figure 4 E, three arrows)**. Quantification of collateral development after seven days was performed by counting the number of collaterals larger than 1 mm in diameter and plotting them on a correlation graph (**Figure 4E**). There is a good correlation

(R=0.83, p<0.001) between the number of collaterals > 1mm and the volume increase in volume of the portal vein supplied sector within seven days.

*Outcome: Hemodynamic changes in PVL and PVL+HVL*

Portal vein volume flow measurements prior to and 1 hour after performing the ligations of PVL or PVL+HVL, showed no significant difference in main portal vein flow after one hour or seven days later, when the livers were procured. This indicates that in pigs, the same mesenteric blood volume is pushed through the portal supplied part of the liver after the ligation procedures as is flowing through the entire liver before, as has been demonstrated in previous experiments in our laboratory in rats[8] and pigs.[7] The index of volume of portal blood flow (in ml) per volume liver tissue (in cc) per minute increased from 1.06 (IQR0.83-1.25) and 1.29 (IQR 1.14-1.36) ml/cc/min to 3.69 (IQR2.93-5.39) and 5.68 (IQR4.61-5.96) ml/cc/min in PVL and PVL+HVL, respectively after the ligation procedures on day 0. There is no difference in the flow changes between PVL and PVL+HVL. The index remains elevated after 7 days, if the growing portal vein supplied liver remnant is in the denominator as shown in **Figure 5A**. If the portal blood however perfuses a larger part of the liver as suggested by the collaterals found in the epoxy casts, the denominator increases in a way that is difficult to determine and the true index is difficult to assess. The gradient between central vein pressure (CVP) and portal vein pressure in pigs ("portacaval gradient") in mmHg at baseline is 1 (IQR1-2.75) mmHg and 2.5 (IQR1.75-3) mmHg and increases to 6.5 (IQR5.25-7.75) mmHg and 5.5 mmHg (IQR4.75-9.25) for PVL and PVL+HVL, respectively (p=0.008 and p= 0.007, *resp*.) after the flow restriction to about 21% of the previous liver volume (**Figure 5B**). The acute increase of the pressure gradient returns to normal after 7 days in both procedure types. There is no difference in the acute portal hypertension induced by PVL and PVL+HVL.

*Outcome: Serum hepatocyte injury markers and liver function tests*

The animals were well, no animal died prematurely and no animal had ascites on re-exploration . The deportalized and the growing liver looked viable on re-exploration on day 7 in both groups. **Figure 5C** shows that aspartate aminotransferase (AST) levels are increased (p=0.004) after both PVL and double ligation PVL+HVL, remain so until day 3, when they return to normal. There is no significant difference between PVL and PVL+HVL. Alanine aminotransferase (ALT) remains unchanged and does not differ between procedures **(Figure 5D)**. The liver transfer function metrics total bilirubin (tBili) is very low in pigs at baseline and remains unchanged **(Figure 5E)**. Liver synthetic function metrics International Normalized Ratio (INR) and PT also remained unchanged and does not differ between procedures **(Figure 5F)**.

*Outcome: Histologic changes*

**Figure 6A** shows the normal portal tract anatomy in pigs with normal sized portal veins. **Figure 6B** and **Figure 6C** show dilated portal veins 7 days in the portal vein supplied growing lobes after PLV and PVL+HVL respectively. Quantitative evaluation of Ki-67 staining at baseline **(Figure 6D)**, 7 days after PVL **(Figure 6E),** and 7 days after PVL+HVL **(Figure 6F)** show no significant changes **(Figure 6G)**. The liver lobe deprived of portal inflow and venous outflow does not show any signs of tissue degeneration in 100x magnification **(Figure 6H)** or inflammatory infiltrates or cellular degeneration in 600x magnification **(Figure 6I)**.

**Discussion**

This study demonstrates in a preclinical animal model that the simultaneous double ligation of portal and hepatic veins induces three times the amount of liver hypertrophy compared to portal vein ligation alone within 7 days. This simultaneous double ligation maneuver is surprisingly well tolerated, does not lead to excessive transaminase elevation when compared to portal vein ligation alone and does not produce any laboratory evidence of liver dysfunction as evidenced by serum tBili or INR. Despite the outflow obstruction of all major veins, there is no evidence of hepatocyte necrosis in histology, likely due to remaining arterial flow. The amount of hypertrophy induced by this double ligation technique is at least as extensive as the rapid hypertrophy in a pig model of ALPPS with 64 % volume increase within 7 days.[7] Both methods lead to comparable volume increase in the portal vein supplied sector compared to PVL alone.

The mechanism of the observed effect of double ligation on hypertrophy remains unclear. We speculate that the inflow of hepatotrophic growth factors through collaterals can be abrogated either by transection of the parenchyma or by additional ligation of the hepatic veins additional to the portal veins. We propose that the mechanism is abrogation of collateral flow bringing the substances that have in the past been described as the "hepatotrophic growth factors" like insulin, insulin-like growth factor, epithelial growth factors etc. to the growing liver.[9] In the ALPPS model in pigs the area of portal vein perfusion in ALPPS after 7 days as indicated by methylene blue coloring had been confined to the right lobe along the line of parenchymal transsection (not shown).[7] Similarly now, 7 days after PVL+HVL, the area of portal vein perfusion stayed restricted to too the right lobe **(Figure 3C)** while it dissipated across the entire liver in PVL **(Figure 3B).**

The hypertrophy of the double ligation approach in a preclinical pig model is surprising, since sequential hepatic vein occlusion has in the past been found to be only moderately effective, likely because hepatic vein occlusion has been used previously as a salvage maneuver in a sequential fashion after portal vein occlusion.[10, 11] The profound effect on volume may be the result of the simultaneous occlusion of both systems.[12, 13] It may be that an initial growth stimulus matters without portal escape collaterals matter and sequential embolization does not provide that. Interestingly also, simultaneous portal vein and hepatic vein occlusion has been attempted in preclinical rabbit model in the past, but was found to yield a hypertrophy response that was not considered superior to portal vein embolization alone.[14] The findings may be explained by the anatomic specificities of the rabbit, i.e. the almost complete disconnection of the parenchyma in the highly lobulated rabbit liver.[15] The anatomy in the rabbit liver does not lend itself to study the effects of collateral abrogation because the liver is much more extensively lobulated than the pig liver. After embolization or ligation of the left or distal liver the potential for development of collaterals is minimal because there is only a diminutive parenchymal tissue mass connecting the deportalized and the growing liver. The disappointing findings in patients and animal model may have discouraged others from pursuing a simultaneous double occlusion of both portal and hepatic veins.

Recently however, the simultaneous embolization (rather than ligation) of portal and hepatic veins has been described in humans in a pilot series under the name of "liver venous deprivation" technique (LVD) with results that suggest that simultaneous double embolization in humans also induces rapid hypertrophy.[12, 13]

While ALPPS currently yields a lot of attention because it allows induction of rapid hypertrophy prior to resection with the promise to expand resectability of liver tumors with large tumor load,[4, 16, 17] ALPPS as a routine procedure remains controversial.[18-20] ALPPS has a high perioperative morbidity and mortality rate.[21, 22] Recently improvements in outcomes

have been described, both in highly selected patient populations[23] and in prospective series with more restrictive inclusion criteria[24]. There is accumulating evidence however that ALPPS produces a large volume of immature liver tissue in a short period of time that is voluminous but dysfunctional and may put patients at risk of post-hepatectomy liver failure more often than predicted by the large volume increase.[25-29] In contrast, there is now preliminary evidence in three patients that simultaneous double embolization in humans does not induce a function/volume incongruence.[12] It is an interesting finding that rapid hypertrophy comparable in magnitude to ALPPS does not necessarily result in a functional deficit. The understanding of the physiological elements associated with rapid hypertrophy remain an important task to clarify whether the circulating growth factors like Il-6 and TNF-α, the acute portal hypertension or the difference in the extent of abrogation of collaterals ultimately explain the volume increase and also the occurrence of the functional deficit after classic ALPPS and how functionality can me maintained in rapid hypertrophy. This large animal model confirms that rapid volume increase occurs after double ligation and will allow to study questions of volume and function by comparing PVL, the ALPPS model and double ligation..

There is evidence that the number of collaterals negatively correlates with the degree of hypertrophy in PVL and ALPPS.[7] Additionally, there are case reports on failed hypertrophy due to developing porto-portal collaterals after PVE in humans.[30] There also is the presumption that PVE is more effective than PVL in humans because of reduced collateralization in general when the portal venous space is filled with glue.[31] Going back almost 100 years, Peyton Rous observed collaterals to the portal vein deprived liver in the first paper on experimental portal vein ligation in 1921.[32] He noticed that *"The rapidity and*

*completeness of the atrophy is in our experiments proportionate to the number of these little collaterals. Their influence may be directly seen where they enter the liver."*[32]

In the epoxy casts a reduction, not a complete absence of collaterals in the double ligation model was found. Both models induced acute portal hypertension and in both models gradients normalized one week after ligation. It has to be emphasized that hepatic veins, while occluded by US immediately after the performance of PVL+ HVL, had partially recanalized at day 7. These observations warrant future studies on the hemodyamics of collateral flow along the time axis.

The histologic findings of this study and the comparable Ki-67 proliferation rates of hepatocytes are different from our findings in the ALPPS model in pigs[7], and suggest that the process of hepatocyte proliferation in PLV+HVL in pigs is terminated prior to day 7. Evaluations at earlier time points should address this point in the future.

A methodological limitation of this study is the lack of cross-sectional imaging in our large animal facility to allow a detailed diachronous assessment of volume changes and possibly also an interrogation of in vivo inflow kinetics using contrast. Also, an assessment of liver function by regional liver function test using hepatobiliary iminodiacetic acid (HIDA) scanning was not yet possible in this study due to the absence of a gamma-camera or SPECT computer tomography for large animals. Combined volume and function assessment would greatly enhance the understanding of the different types of liver hypertrophy und their functionality. Differently from the human studies on double embolization this large animal model allows an interrogation of the vascular anatomy by performing epoxy vascular casts. These casts provide a static snapshot of anatomic detail of the collaterals developed, that has not been demonstrated by any other study so far.

Another limitation is that PVL+HVL is compared to PVL alone, but not directly also to ALPPS or PVE. In a large animal model the three Rs (reduce, refine, replace) of laboratory

animal science led us to not repeat the previously published ALPPS series again but compare the new results with PVL+HVL to data published about ALPPS in the pig.[7] The allocation to two groups, PVL vs. HVL+PLV is a simple study design. The power analysis for sample size was performed based on data from our previously published study of PVL vs. ALPPS. The randomization resulted in an asymmetric allocation of 8 vs. 6 animals, which is methodologically unavoidable. In the future, PVE in the pig and also hepatic vein embolization in this laboratory will be tested but they require a new experimental set up with fluoroscopy and involvement of interventional radiologists.

In summary, this preclinical study demonstrates the feasibility and safety of simultaneous unilateral occlusion of hepatic and portal veins to induce rapid liver regeneration. It maybe the case that the double venous ligation or embolization technique in man allows for a safer acceleration of hypertrophy than ALPPS has so far. Double ligation and embolization should be further evaluated with careful analyses of liver function to evaluate this potential alternative to ALPPS in regenerative liver surgery.

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## Figure Legends

### Figure 1

**Experimental procedures in pigs. (A) Portal vein anatomy of the pig liver**. The right lateral sector (**RL**), caudate lobe sector(**CL**), right median sector (**RM**), left median sector (**LM**) and left lateral sector (**LL**) are shown. Portal vein supply sectors are not congruent with the borders of the external lobulation, a common theme in liver anatomy. The watersheds between sectors cross the lobes and tissue bridges between lobes in a sigmoid line (dotted line). **(B) Portal vein ligation (PVL) procedure** PVL leads to an acute cessation of portal vein flow to RM, LM and LL and portal hyperflow to RL and CL. The ligation of the portal vein distal to the take-off of the RL lobe branch (arrow) is achieved using portal pedicle dissection and a silk tie. **(C) Portal vein ligation + hepatic vein ligation procedure (PVL+HVL**). In PVL+HVL, additionally to PVL the three hepatic veins draining RM, LM and LL are additionally ligated using long and large XLS needles with Nr. 1 Prolene sutures and pledgets. The RM, LM and LL veins are ligated. **(D) Intraoperative ultrasound** is used to identify the three veins draining RM, LM and LL. A sponge is placed between the right lobe and the right median lobe to delineate a line of echogenicity between the lobes (white arrows) to safely spare the RL vein. (**E) Intraoperative photograph of PVL+HVL**. The photo shows the representative placement of a venous occlusion stitch with the white umbilical tape serving as a pledget. The three inserts show the steps of the procedure, the depth of the needle tract is determined by ultrasound. (**F**) **Photograph of intraoperative ultrasound**. At the end of the PVL and HVL procedure intraoperative ultrasound confirms the occlusion of the RM, LM and LL veins and a patent RL vein that drains into the vena cava.

**Figure 2**

**Volume changes of the pig liver over 7 days after portal vein ligation (PVL) and double ligation of portal vein and hepatic veins (PVL+HVL). (A) Absolute volume changes of the portal vein supplied sector (right lateral and caudate lobe – RL+CL) in cubic centimeters over 7 days.** The volume of the portal vein supplied sector (RL+CL) increases significantly after PVL+HVL (°p< 0.001), but not significantly after PVL (p=0.14). The difference in size between the portal vein supplied lobe 7 days after PVL and 7 days after PVL+HVL is significant (*p<0.001). Data are normally distributed by Kolmogorov-Smirnov test and one-way ANOVA was used for comparison of 4 groups. **(B) Relative volume change in the portal vein supplied sector in percent.** The graph shows the relative volume increase of the portal vein supplied sector after both procedures compared to baseline. **C) Absolute volume changes in portal vein deprived sectors (right median, left median and left lateral sector – RM+LM+LL).** The portal vein deprived liver (RM+LM+LL) reduces significantly in size after PVL+HVL (p<0.001), while it does not change significantly after PLV alone (p=0.10). There is a significant difference between the size of the deportalized sector after PVL and PVL+HVL (p<0.001). Data are normally distributed by Kolmogorov-Smirnov test and one-way ANOVA was used for comparison of 4 groups. **(D) Relative volume change in the portal vein deprived sectors in percent.** The graph shows the collapse of the volume of the portal vein deprived liver sectors after PVL+ HVL when compared to PVL.

**Figure 3 Methylene-blue staining after injection of 10 ml methylene blue into the main portal vein (A) at baseline, (B) 7 days after PVL and (C) 7 days after double ligation of portal vein and hepatic veins**. **(A)** The sigmoid shape of the portal supply area of right lateral and caudate lobe sector (RL+CL) is delineated by the blue staining of the watershed.

**(B)** There is no intraoperative line of demarcation 7 days after PVL since the methylene blue distributes across the entire liver, likely through the collaterals that have developed. **(C)** There is a line of demarcation after PVL +HVL, somewhat shifted to the right median sector when compared to the baseline in (A).

**Figure 4**

**Evaluation of portal vein collaterals** using expoxy casts of the portal vein system (red resin) and the hepatic veins (blue resin) of entire pig livers 7 days after portal vein ligation (PVL) and double ligation of portal and hepatic veins (PVL+HVL) **(A) Epoxy cast of pig liver 7 days after PVL** shows the right lateral sector (RL), the right median sector (RM), the left median (LM) and the left lateral sector (LL) of the pig liver with the blue resin as the hepatic veins and the red resin the portal vein system. There are extensive neocollaterals (red resin) across the normally avascular watershed between the RL and the RM giving rise to secondary filling of the portal vein tree of RM, LM and LL. The vena cava (blue) is deflected posteriorly and marked by asterisk (*). The square frames the enlarged area in Figure 4 (B). **(B) The enlargement of the frame in 4A** shows the 3 main groups of collaterals observed in the PVL cast, (one arrow, red) anterior to the RL vein, (two arrows, red ) posterior to the RL vein and (three arrows, red) through the caudate lobe. **(C) Epoxy cast of pig liver 7 days after PVL+HVL.** The extensive neocollateralization between RL and RM observed after PVL is here notably absent except for very few collaterals. The vena cava (blue) is deflected inferiorly and marked by asteriks (*). The 3 pledgets made from umbilical tape and used to reinforce the venous ligation stitches on the liver surface are visible even after the digestion process of the liver tissue. The ligated hepatic however have recanalized. The square frames the enlarged area in figure 4D. **(D) The enlargement of the frame in 4C** shows one large > 1mm diameter neocollateral (red) giving off multiple small branches (one arrow). There is

also a group of collaterals posterior to the RL hepatic vein (red, two arrows). **(E) The caudate lobe collaterals 7 days after PVL+HVL** are best visualized from an inferior perspective (red, three arrows) **(F) Correlation graph of collaterals measured by a Vernier digital microcaliper to be >1mm and percent volume increase** in 4 epoxy casts from pig livers 7 days after PVL (yellow) and 6 epoxy casts from pig livers 7 days after PVL+HVL (blue). There is a significant negative correlation (glomerular tufts = - 0.1605 * % hypertrophy + 18.99) with a $R^2$ of 0.83.

**Figure 5**

**Hemodynamic and laboratory changes after Portal Vein Ligation (PVL) and Double Ligation of Portal Veins and Hepatic Veins. (A) Volume flow in the main portal vein** corrected for the volume of liver tissue perfused. Intraoperative direct measurements of main portal vein flow corrected for the estimated tissue volume perfused based on estimated liver weight shows an increase in portal vein flow per tissue immediately after the procedure for both PVL (p=0.017) and PVL+HVL (p= 0.002). There are no differences between PVL and PVL+HVL for all measurements. The perfused volume after 7 days was the weighed hypertrophied liver. Given the findings of collaterals which may well recruit portal volume flow to the side that was deprived of portal flow during the procedure, a correct estimation the perfused volume at day 7 is difficult. **(B) Pressure gradient between in the main portal vein and the inferior vena cava** was measured intraoperatively by using 18 gauge intra-portal needles. The results demonstrate an acute portal hypertension as a result of both devascularization procedures PVL and PVL+HVL. In both procedures the portocaval gradients return to normal after 7 days. Friedman test for repeated measurements of non-parametric data is used, Kruskal-Wallis test is used for comparison of groups. **(C) Asparatate transferase (AST)** levels before and after the procedure at day 0, at day1 and every other day

until day 7. There is no significant increase on day 0 after PVL, but after PVL+HVL ($p<0.001$), but no difference between groups. **(D) Alanine transferase (ALT)** levels. **(E) Total bilirubin (tbili)** levels. **(F) International Normalized Ratio (INR)** levels. There is no significant difference between time points or group for ALT, AST, tbili or INR. For all statistics in this figure, Friedman test for repeated measures of non-parametric data was used. Kruskal-Wallis test was used for comparison of groups.

**Figure 6**

**Histology 7 days after Portal vein ligation (PVL) and double ligation of portal veins and hepatic veins (PVL+HVL). (A) Normal portal triad of a pig liver** at day 0 prior to surgery shows normal sized portal vein (arrow), artery and bile duct (x200 magnification). **(B) Portal triad 7 days after PVL** shows dilated portal veins (arrows) with normal bile ducts and artery (x200 magnification). **(C) Portal triad 7 days after PVL+HVL** shows a dilated portal vein (arrow) (x200 magnification). **(D) Ki-67 immunohistochemistry of a normal pig liver** at day 0 prior to surgery with staining of hepatocytes (arrow), occasional Kupffer cells (short arrow) (x400 magnification). **(E) Ki-67 immunohistochemistry 7 days after PVL** with staining of hepatocytes (arrow), occasional Kupffer (short arrow) and endothelial cells (thin arrow) (x400 magnification). **(F) Ki-67 immunohistochemistry 7 days after PVL+HVL** with a very similar appearance, staining of hepatocytes (arrow), occasional Kupffer (short arrow) and endothelial cells (thin arrow) (x400 magnification). **(G) Quantitative evaluation of Ki-67 staining between PVL and PVL+HVL.** No difference was detected. Friedman test for repeated measures of non-parametric data was used. Kruskal-Wallis test was used for comparison of groups. **(H) No evidence of necrosis** or degeneration of histologic architecture in the portal vein and hepatic vein deprived lobe 7 days after PVL+HVL (magnification x100) **(I) No evidence of necrosis**, cytoplasmic degeneration or inflammation 7 days after

PVL+HVL in higher magnification x600. *Each bar in the lower right corner represents 100 microns which was calibrated according to magnification using ImageJ*.

**Figure 1**

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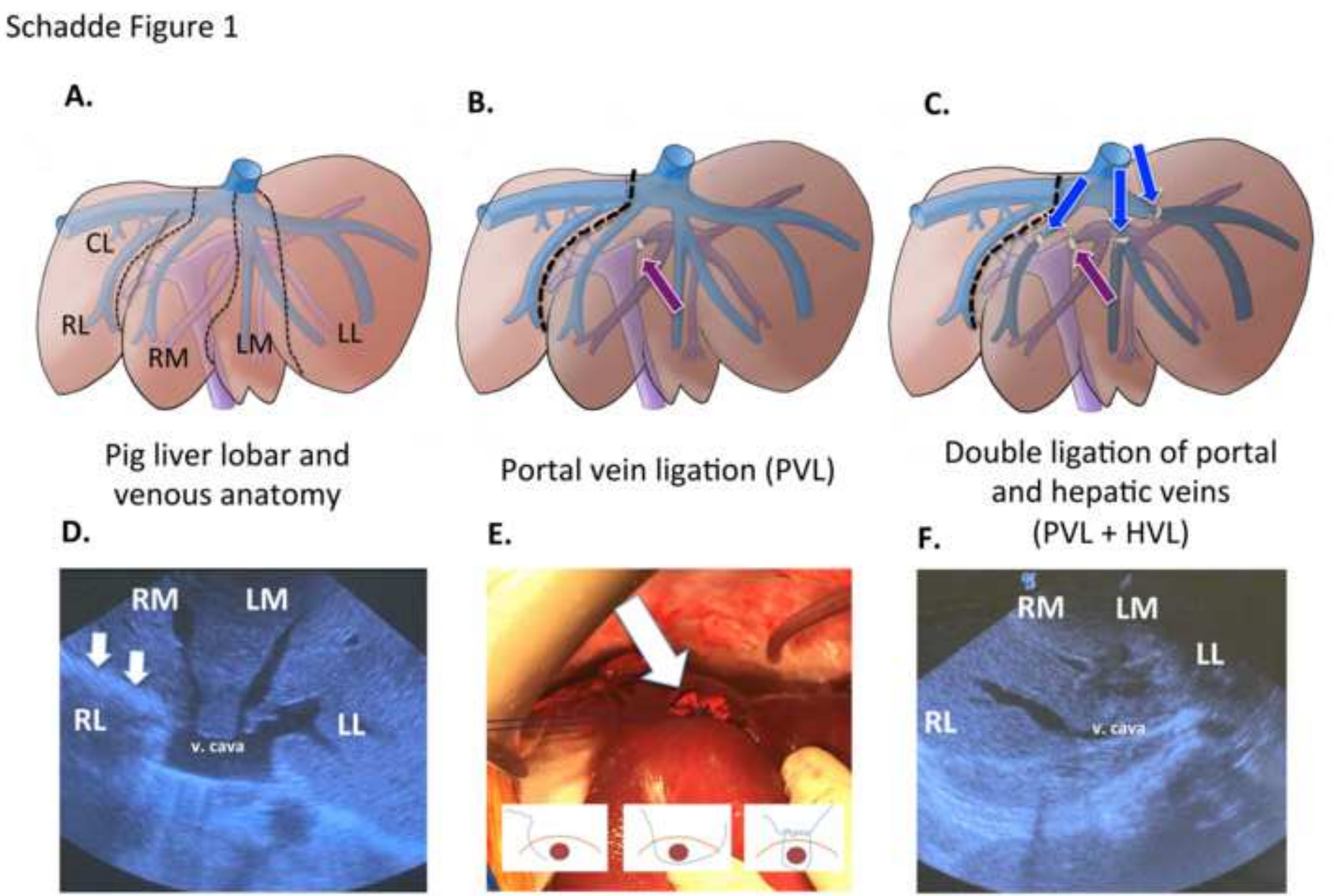

**Figure 2**
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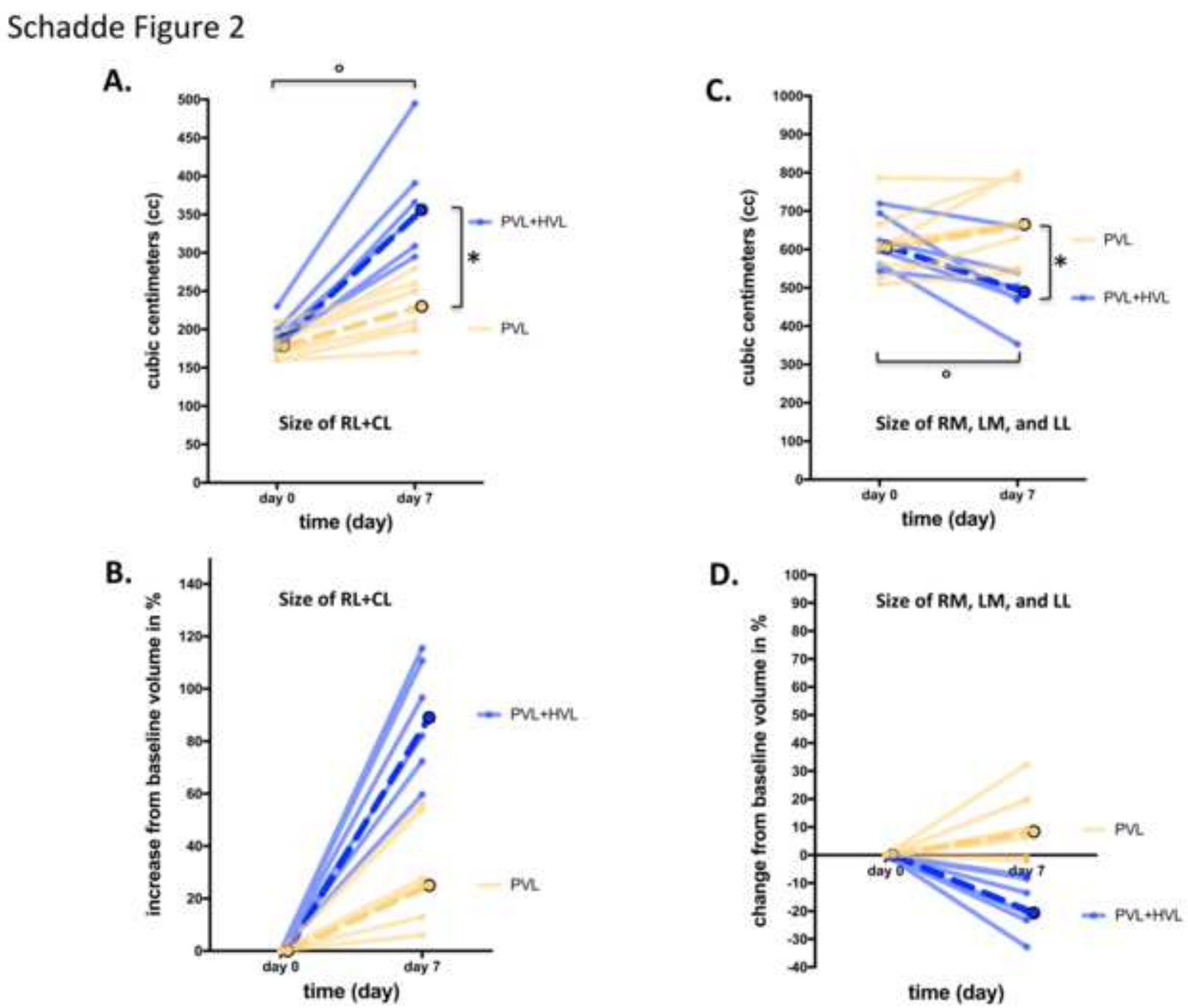

**Figure 3**

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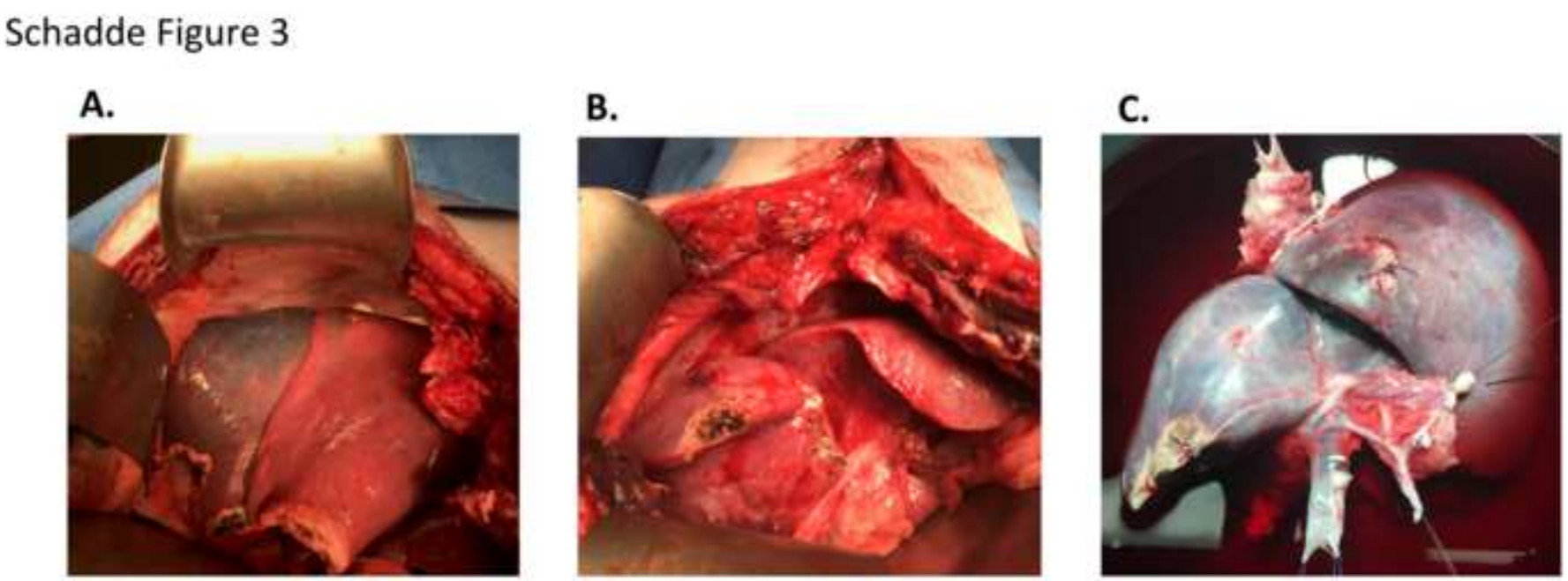

**Figure 4**
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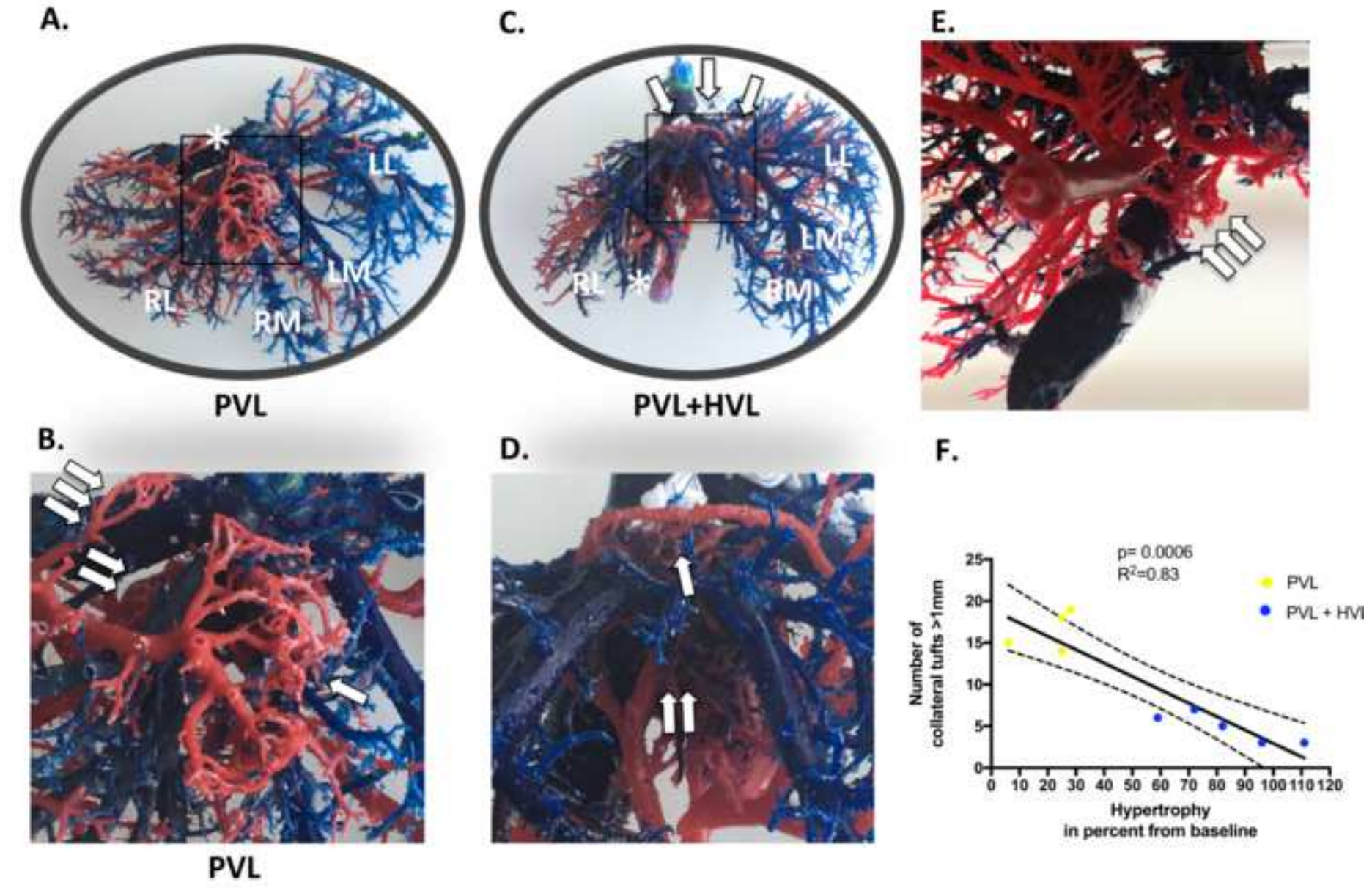

**Figure 5**

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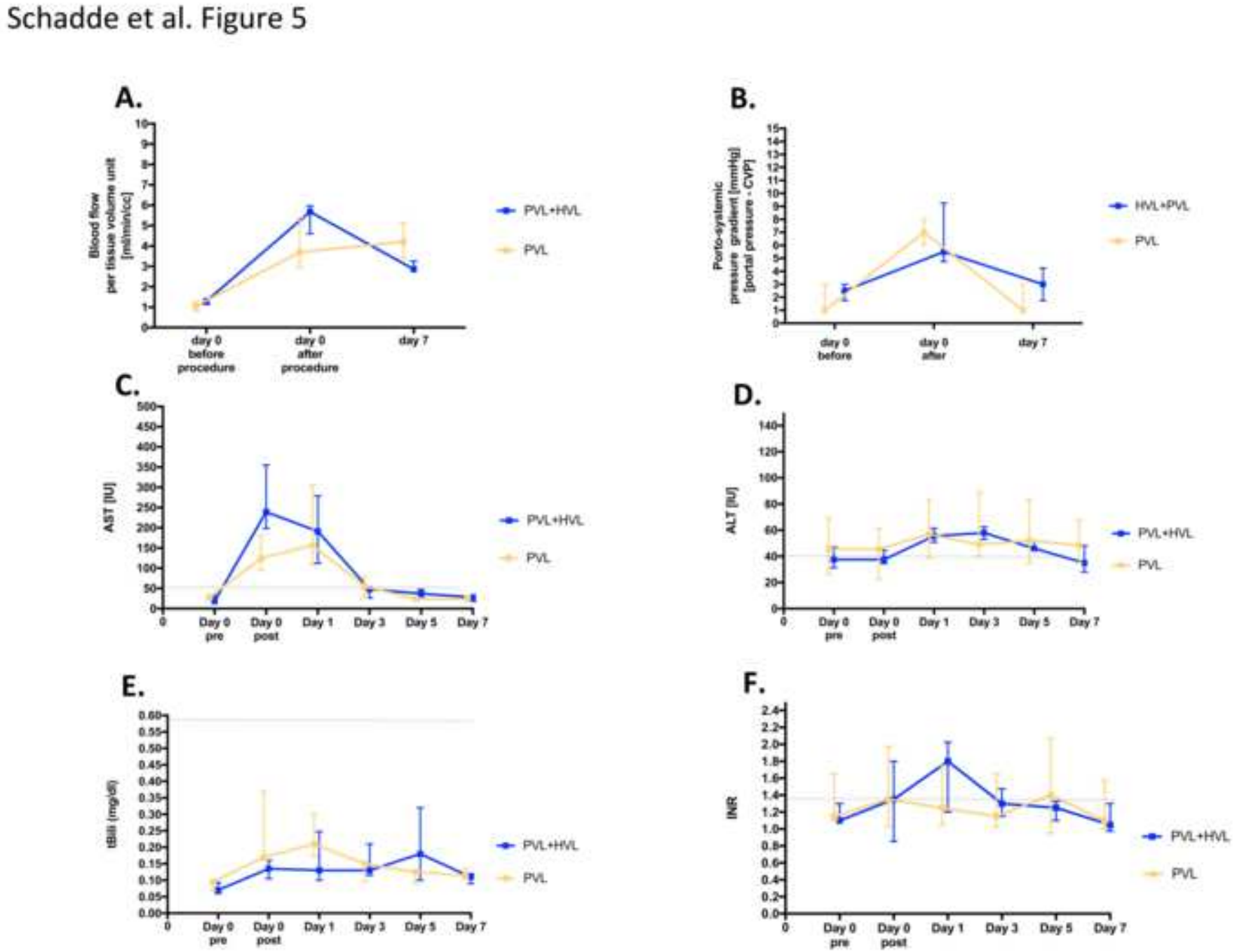

**Figure 6**
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